PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : G01N 33/53

A1

(11) International Publication Number: WO 00/62062

(43) International Publication Date: 19 October 2000 (19.10.00)

(21) International Application Number: PCT/KR00/00329

(22) International Filing Date: 10 April 2000 (10.04.00)

(30) Priority Data:

1999/12568	9 April 1999 (09.04.99)	KR
1999/43935	12 October 1999 (12.10.99)	KR

(81) Designated States: AU, BR, CA, CN, ID, IN, JP, MX, NZ, RU, SG, TR, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

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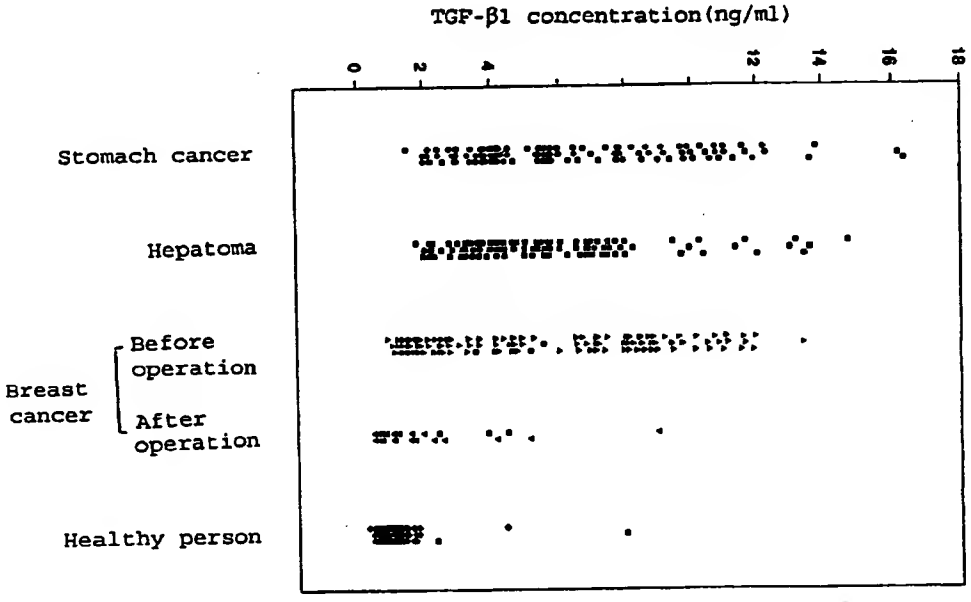
(54) Title: METHOD FOR QUANTIFYING TRANSFORMING GROWTH FACTOR–β1 AND METHOD FOR DETECTING CANCER BY USING SAME



(57) Abstract

The amount of TGF–β1 in a sample is quantified by treating the sample with a TGF–β1 specific receptor to from a complex between TGF–β1 and the receptor and measuring the amount of the complex.

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METHOD FOR QUANTIFYING TRANSFORMING GROWTH FACTOR-β1 AND METHOD FOR DETECTING CANCER BY USING SAME

FIELD OF THE INVENTION

The present invention relates to a method for quantifying the concentration of transforming growth factor-β1(TGF-β1) in a body fluid, a method for detecting cancer by using same, a composition for detecting cancer, and a TGF-β1-specific monoclonal antibody.

BACKGROUND OF THE INVENTION

Transforming growth factor-β(TGF-β) regulates the growth and differentiation of several cells, its mode of action depending on the cell configuration and the presence of other growth factors(Sporn et al., *Science*, 233, 532-534 (1986); and Roberts and Sporn, *Adv. Cancer Res.*, 51, 107-145 (1988)).

Three forms of TGF-β factor, TGF-β1, -β2 and -β3, occur in mammals, and, among these, TGF-β1 is believed to play a key role in the physiological mechanism and disease progression. It has been reported that it acts abnormally in an invasion process, e.g., carcinogenesis. This suggests that TGF-β1 is useful as a tumor marker in cancer diagnosis, and that a method for quantifying TGF-β1 in a body fluid with a high precision can be critical in cancer diagnosis.

EP Publication No. 0 722 773 A1 discloses a method for detecting cancer by contacting a blood sample containing TGF-β1 with an absorbent, OH-carbonated hydroxyapatite, to adsorb TGF-β1 thereto, eluting the absorbed TGF-β1 with a buffer, and determining the amount of TGF-β1 eluted with UV spectrometry. However, this method suffers from the problems of limited

sensitivity and imprecision manifested by large fluctuations in measured values.

Therefore, there has existed a need to develop an improved method for quantifying the amount of TGF-β1 in plasma.

SUMMARY OF THE INVENTION

Accordingly, it is an object of the present invention to provide a method for quantifying the amount of TGF-β1 in a sample with high precision and sensitivity.

Another object of the present invention is to provide a method for detecting cancer by using said method.

A further object of the present invention is to provide a composition for detecting cancer.

A still further object of the present invention is to provide a TGF-β1-specific monoclonal antibody and a hybridoma cell line producing the monoclonal antibody.

In accordance with one aspect of the present invention, there is provided a method for quantifying the amount of TGF-β1 in a sample which comprises treating the sample with a TGF-β1-specific receptor to form a complex between TGF-β1 and the receptor and measuring the amount of the complex.

BRIEF DESCRIPTION OF THE DRAWINGS

The above objects and features of the present invention will become apparent from the following description of preferred embodiments taken in conjunction with the accompanying drawings, in which:

Fig. 1 shows the optical density-TGF-β1 concentration correlations obtained in Example 1 for TGF-β1 type III and type II receptors, respectively;

Fig. 2 depicts the respective distributions of TGF-β1 concentrations in plasma samples taken from healthy persons, stomach cancer patients, hepatoma patients and breast cancer patients; and

Fig. 3 provides the respective distributions of TGF-β1 concentrations in plasma samples taken from healthy persons, lung cancer patients, rectal-colic cancer patients, prostate cancer patients and uterine cervical cancer patients.

DETAILED DESCRIPTION OF THE INVENTION

The TGF-β1-specific receptors which may be used in the present invention include TGF-β1 type I, II and III receptors(RI, RII and RIII), and preferred is TGF-β1 type III receptor, RIII. The TGF-β1 receptors may be obtained by expressing a TGF-β1 receptor gene in a mammal or insect cell line in accordance with a conventional method(Burand, J.P. et al., *Virology* 101, 286-290 (1980)). For example, the TGF-β1 receptor may be obtained by infecting an insect cell line, e.g., Sf21(Invitrogen, Netherlands), with a recombinant baculovirus containing a TGF-β1 receptor gene; extracting a water-insoluble receptor protein expressed in the insect cell; solubilizing the water-insoluble receptor protein with guanidine HCl or urea; refolding the solubilized receptor protein by removing the guanidine or urea to restore the affinity for TGF-β1.

The TGF-β1-specific antibody which may be used in the present invention may be prepared by immunizing a mammal with TGF-β1 or a part thereof. The TGF-β1-specific antibody may be a monoclonal antibody or a polyclonal antibody having a specificity only for TGF-β1.

A preferred method for quantifying the amount of TGF-β1 in a body fluid, e.g., plasma or urine, in accordance with the present invention comprises

(a) attaching a TGF-β1-specific receptor to a solid support;

(b) adding a body fluid sample to the supported receptor to form a TGF-β1-receptor complex;

(c) binding a TGF-β1-specific antibody conjugated with a label to the complex; and

(d) measuring the amount of TGF-β1 using the label as a detection marker.

Representative labels which may be employed in the present invention include horseradish peroxidase, biotin and fluorescence.

A first preferred embodiment of the present invention comprises attaching a TGF-β1 receptor to a solid support, e.g., the well of a microtiter plate; adding an appropriately diluted sample containing TGF-β1 to the TGF-β1 receptor to allow the formation of a complex between TGF-β1 and the TGF-β1 receptor; washing the support with a phosphate buffered saline(PBS); adding thereto a chromogenic enzyme-conjugated anti-TGF-β1 antibody and developing the chromogenic enzyme; and measuring the optical density of the resulting solution to quantify the content of TGF-β1 in the sample.

In a second preferred embodiment of the present invention, a liquid containing a TGF-β1-specific receptor may be used in place of the supported TGF-β1 receptor. In this method, the amount of TGF-β1 in a sample may be quantified by adding the sample to the liquid containing a TGF-β1-specific receptor; adding a TGF-β1 specific antibody conjugated with a label thereto; precipitating an antibody-TGF-β1-receptor complex; and measuring the optical density thereof.

The inventive method is capable of detecting TGF-β1 at a very low concentration range of 30 pg/ml or below.

The above method is particularly useful in cancer diagnosis, since the TGF-β1 concentration in a cancer

patient's body fluid is distinctly different from that of a healthy person. Accordingly, a cancer may be detected by repeating the above method to quantify the TGF-β1 level in a patient's body fluid sample, e.g., plasma or urine; and comparing the TGF-β1 concentration with that of a healthy person.

A preferred embodiment of the inventive method for detecting a cancer comprises

(a) attaching a TGF-β1-specific receptor to a solid support;

(b) adding a body fluid sample to the supported receptor to form the TGF-β1-receptor complex;

(c) binding a TGF-β1-specific antibody conjugated with a label to the complex;

(d) measuring the amount of TGF-β1 using the label as a detection marker; and

(e) comparing the TGF-β1 amount with that of a healthy person.

The above method is particularly effective in detecting stomach cancer, hepatoma, breast cancer, lung cancer, rectal-colic cancer, prostate cancer and uterine cervical cancer.

A composition which may be used in the method for detecting a cancer comprises a TGF-β1 receptor, preferably RIII, and a TGF-β1 specific antibody.

In order to improve the sensitivity, the monoclonal antibody may be obtained by preparing a hybridoma cell line which produces TGF-β1-specific monoclonal antibody using TGF-β1 or an antigenic determinant part thereof as an immunogen according to a conventional cell fusion method; and isolating the monoclonal antibody from the hybridoma cell line. For example, such a hybridoma cell line may be prepared by immunizing a mouse with human TGF-β1; fusing the mouse spleen cell with myeloma cell according to the cell fusion method described by Kohler and Milstein(*Eur. J.*

Immunol., 6, 511-519 (1976)); selecting by way of using ELISA a hybridoma cell line having a specificity only for human TGF-β1; determining the subclass of the monoclonal antibody produced by the hybridoma cell line using an immunodiffusion method; and selecting a hybridoma cell line secreting IgG1 subclass with the highest antibody titer. The hybridoma cell line thus obtained was designated hTGF-46 and deposited with Korean Collection for Type Culture(Address: #52, Oun-dong, Yusong-ku, Taejon 305-600, Republic of Korea) on April 20, 1998 under the accession number of KCTC 0460BP, in accordance with the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganism for the Purpose of Patent Procedure.

Hybridoma cell line hTGF-46 originates from β-limphoma, and continuously divides while producing human TGF-β1-specific, IgG1 subclass antibody. The hybridoma cell line may be cultured in RPMI 1640 medium(Gibco-BRL, USA) containing 10% bovine fetal serum at 37 °C under an atmosphere of 5% CO_2 and 100% humidity. The cell number doubles in 12 to 14 hours. This hybridoma cell line floats on the medium without attaching itself to the bottom of the culture flask and has a round shape having a diameter of 15 to 20 μm.

To produce a large amount of the TGF-β1-specific monoclonal antibody from the hybridoma cell line, the hybridoma cell line is injected to a mouse and when its abdominal cavity swells the ascites containing a high concentration of hybridoma cells is taken to isolate the monoclonal antibody therefrom.

When TGF-β1, -β2 and -β3 are subjected to electrophoresis followed by western blotting, the monoclonal antibody of the present invention recognizes only TGF-β1, but not TGF-β2 or -β3. This suggests that the present monoclonal antibody has a unique specificity for TGF-β1. The monoclonal antibody of the present

invention also shows a high affinity toward human TGF-β1, and binds to the epitope region corresponding to the 5th to 80th amino acid residues of TGF-β1.

The following Examples are intended to further illustrate the present invention without limiting its scope.

Further, percentages given below for solid in solid mixture, liquid in liquid, and solid in liquid are on a wt/wt, vol/vol and wt/vol basis, respectively, unless specifically indicated otherwise.

Example 1: Sensitivity of TGF-β1 for Receptor

(Step 1) Preparation of TGF-β1 type III receptor

Plasmid pCEP4(Invitrogen, Netherlands) containing a full length cDNA of Human TGF-β1 type III receptor was subjected to polymerase chain reaction(PCR) using primers RIII1 and RIII2(SEQ ID NOs: 1 and 2) to obtain a DNA fragment encoding an extracellular domain of the receptor which is composed of 400 amino acid residues(1 to 400). The DNA fragment thus obtained was inserted into baculovirus vector pCRBac(Invitrogen, Netherlands) to obtain recombinant plasmid pCRBac-TGFR. *E. coli* cells were transformed with the recombinant plasmid pCRBac-TGFR and the transformed *E. coli* cells were selected on a selective medium, LB medium containing ampicillin.

Vector pCRBac-TGFR and Bac-N-Blue DNA(Invitrogen, Netherlands) were cointroduced to insect cell line Sf-21(Invitrogen, Netherlands) using the liposome transfection method(Burand, J.P., *Virology*, 101, 286-290 (1980)) and cultured for 3 days to obtain a virus product. After 3 days, the virus thus obtained was subjected to plaque analysis using lacZ gene as a selective marker to select the recombinant virus. The

recombinant virus thus obtained was subjected to PCR using forward primer(SEQ ID NO: 3) and reverse primer(SEQ ID NO: 4) to confirm the presence of the TGF-β1 receptor gene. The wild vaculovirus showed a 839 bp PCR product whereas the recombinant virus gave a 1.5 kbp PCR product.

Insect cell line SF21 was infected with the recombinant virus and then cultured for 5 days. The culture was centrifuged to remove cell debris and the supernatant containing virus was collected.

Insect cell line Sf21 was inoculated with the supernatant and then cultured at 27 °C for 72 days in Grace Insect medium(Invitrogen, Netherlands) containing 10% fetal bovine serum(FBS), 7.3% TC yeastolate, and 73% lactoalbumin hydrolysate. The culture was centrifuged to collect cells and the cells were washed with PBS. Protein lysis solution(50 mM Tris-HCl(pH 7.5), 50 mM NaCl, 10 mM β-mercaptoethanol, 1% Triton X-100 and 2 mM BMSF) was added thereto and then the resulting solution was heated at 100 °C for 10 minutes to prepare a sample.

The sample was subjected to SDS-PAGE in 12.5% SDS-polyacrylamide gel and the resulting gel was stained with coomassie brilliant blue. The gel was subjected to western blotting which was conducted by electrically transferring the proteins separated on the gel to a filter, binding the antibody for TGF-β1 receptor obtained from R&D Systems Inc., USA to the proteins of the filter and then analyzing the TGF-β1 using horseradish peroxidase(HRP)-conjugated anti-IgG secondary antibody(Chemicon, USA) to confirm the expression of the TGF-β1 III receptor.

Since the TGF-β1 receptor is water-insoluble, 8M guanidine HCl(pH 8.0) was added to the sample and the resulting solution was stirred for 1 hour. The resulting solution was centrifuged at 7,000 rpm for 40

minutes and then the supernatant was adjusted to a protein concentration of 2 mg/ml. To restore the binding activity of the TGF-β1 receptor to TGF-β1, the resulting solution was added to a refolding buffer(100 mM Tris, 0.5 M arginin, 0.2 M EDTA, pH 8.0) to a protein concentration of 150 μg/ml and kept at 10 °C for 40 hours. The resulting solution was dialyzed with 20 mM Tris solution(pH 8.0), successively in the order of twice every 4 hours, once after overnight, and twice every 2 hours thereafter, to effectively refold the TGF-β1 receptor.

(Step 2) Sensitivity of TGF-β1 type III receptor for TGF-β1

Each 2 μg of the TGF-β1 type III receptor obtained in Step 1 was placed in the wells of a microtiter plate and the resulting plate was held at an ambient temperature for 24 hours to attach the receptor on the plate. 2 ng of purified TGF-β1(R&D systems Inc., USA) was dissolved in PBS and diluted serially. Each dilution solution was added in an amount of 100 μl to the well and then held at an ambient temperature for 3 hours to allow the TGF-β1 bind the receptor. Each well was washed with PBS containing 0.05% of Tween 20(PBST) and then HRP-conjugated anti-TGF-β1 antibody(R&D systems Inc., USA) was added thereto. The resulting plate was left at room temperature for 1.5 hours. Each well was washed with PBST. 100 μl of TMB-ELISA(Gibco-BRL, USA), a substrate of HRP, was added thereto and the resulting plate was left at an ambient temperature for 20 minutes to develop. The development reaction was terminated by adding 25 μl of 2N sulfuric acid. The optical density of the reaction mixture was determined at a measuring wavelength of 450 nm and a correction

wavelength of 570 nm, and the result were plotted to obtain a standard optical density-concentration correlation.

Fig. 1 shows that the correlation thus obtained is a straight line with a correlation coefficient of 0.999 and a slope of 0.28. The slope represents the sensitivity of the receptor used in the measurements and the TGF-β1 type III receptor is deemed to have an excellent sensitivity toward TGF-β1 binding. The correlation in Fig. 1 also shows that an extremely low concentration of TGF-β1, down to 10 pg/ml, can be detected by the present method.

The above procedure was repeated using TGF-β1 type II receptor(R&D systems Inc., USA) to determine the sensitivity of the TGF-β1 type II receptor. The results which are also plotted in Fig. 1 show that the correlation obtained using TGF-β1 type II receptor is also a straight line with a correlation coefficient of 0.999 and a slope of 0.57. Accordingly, the type II receptor may also be used in quantifying the concentration of TGF-β1 but its sensitivity is considerably lower than that of type III receptor.

Example 2: Specificity of TGF-β1 Type III Receptor for TGF-β1

The procedure of step 2 of Example 1 was repeated except that a mixture containing 2,000 pg/ml TGF-β1, 2,000 pg/ml TGF-β2 and 2,000 pg/ml TGF-β3(R&D Systems Inc., USA) was used in place of TGF-β1. The procedure of step 2 of Example 1 was repeated using 2,000 pg/ml TGF-β1 as a control. Results are shown in Table I.

Table I

	TGF-β1	TGF-β1 + TGF-β2 + TGF-β3
TGF-β1 III receptor	100%	91.5%

As can be seen from Table I, the TGF-β1 type III receptor binds only with TGF-β1 without the occurrence a crossreaction with TGF-β2 or TGF-β3.

Example 3: Measurement of Plasam TGF-β1 Concentration in Cancer Patients Using Monoclonal Antibody

Blood samples were taken from 101 healthy persons, 111 stomach cancer patients, 100 hepatoma patients and 151 breast cancer patients. Blood samples were collected with vacuumtainer containing 0.081 ml of 15% ethylene diamine tetraacetic acid(EDTA) as an anticoagulant, and then the resulting mixture was centrifuged at 3,000 rpm for 20 minutes to obtain a plasma sample. 0.1 ml of the plasma sample was added to 0.1 ml of 2.5 N acetic acid/10 M urea solution. The resulting mixture was kept at room temperature for 10 minutes, and neutralized with 0.1 ml of 2.7 N NaOH containing 1M hydroxyethyl piperazine ethanesulfonic acid(HEPES). Activated plasma thus obtained was diluted 4-fold with PBST to obtain a plasma sample solution which was subjected to the following process for measuring its TGF-β1 concentration.

0.1 ml of the plasma sample solution thus obtained, as well as 0.1 ml portions of TGF-β1 standard solutions(0, 100, 1,000 and 2,000 pg/ml), were respectively added to the wells of a 96-well plate containing TGF-β1 type III receptor, kept at room temperature for 3 hours, and then, washed three times with PBST. Purified TGF-β1 monoclonal antibody-HRP complex(Sigma) was added to each well and then the

plate was kept at room temperature for 1.5 hours, followed by washing the wells three times with PBST. 100 μl of TMB ELISA(Gibco-BRL, USA), a substrate of HRP, was added to each well and the plate was kept at room temperature for 20 minutes to develop. The development reaction was terminated by adding 25 μl of 2N sulfuric acid. The optical density of the reaction mixture was determined at a measuring wavelength of 450 nm, and a correction wavelength of 570 nm. The TGF-β1 concentration of the plasma sample was determined based on the calibration curve obtained using standard solutions, and the results are shown in Table II and Fig. 2.

Table II

Plasma Sample Group		Mean ± Standard Error (ng/ml)	Range (ng/ml)
Stomach Cancer(n=111)		6.53 ± 0.31	1.5 - 16.35
Hepatoma(n=100)		5.89 ± 0.3	1.77 - 14.76
Breast Cancer	Before Operation (n=117)	5.49 ± 0.32	0.87 - 13.44
	After Operation (n=34)	2.15 ± 0.42	0.46 - 9
Healthy Person(n=101)		1.03 ± 0.08	0.27 - 8

As can be seen in Fig. 2 which depicts distribution patterns of plasma TGF-β1 concentrations of respective patient groups, the plasma samples of cancer patients, display TGF-β1 concentration patterns which are distinctly different from that of the healthy group. This suggests that the above-mentioned cancers can be detected by measuring plasma TGF-β1 concentration in accordance with the above procedure.

Example 4: Measurement of Plasma TGF-β1 Concentration of Cancer Patient Using Monoclonal Antibody

The procedure of Example 3 was repeated using blood samples taken from 288 healthy persons, 29 lung cancer patients, 48 rectal-colic cancer patents, 50 prostate cancer patients and 88 uterine cervical cancer patients to measure respective plasma TGF-β1 concentrations and the results are shown in Table III and Fig. 3.

Table III

Plasma Sample	Mean ± Standard Error(ng/ml)	Standard Deviation
Lung Cancer(n=29)	8.48 ± 1.27	4.16
Rectal-colic Cancer(n=48)	5.19 ± 0.87	3.69
Prostate Cancer(n=50)	4.12 ± 0.53	2.30
Uterine Cervical Cancer (n=88)	8.55 ± 0.92	5.25
Healthy Person(n=288)	1.17 ± 0.05	0.55

P<0.01

As can be seen in Fig. 3 which shows distribution patterns of plasma TGF-β1 concentrations of respective patient groups, the plasma samples of cancer patients, display TGF-β1 concentration patterns which are distinctly different from that of the healthy group. This suggests that the above-mentioned cancers can be detected by measuring plasma TGF-β1 concentration in accordance with the above procedure.

Example 5: Measurement of Plasma TGF-β1 Concentration of Cancer Patient Using Polyclonal Antibody

The procedure of Example 3 was repeated using blood samples taken from 50 healthy persons, 50 hepatoma

patients and 50 breast cancer patients, except that a polyclonal antibody was used in place of the monoclonal antibody, to measure respective plasma TGF-β1 concentrations and the results are shown in Table IV.

Table IV

Plasma Sample	Mean ± Standard Error(ng/ml)	Standard Deviation	Range (ng/ml)
Hepatoma (n=50)	5.14 ± 0.57	2.92	1.44-16.96
Breast Cancer (n=50)	5.31 ± 0.46	1.67	2.07-10.27
Healthy Person (n=50)	1.19 ± 0.08	0.29	0.70-1.9

$P<0.05$

As can be seen in Table IV, the plasma samples of cancer patients display TGF-β1 concentration patterns which are distinctly different from that of the healthy group. This suggests that the above-mentioned cancers can be detected by measuring plasma TGF-β1 concentration in accordance with the above procedure.

Example 6: Preparation of Hybridoma Cell Line Producing a Monoclonal Antibody for TGF-β1

(Step 1) Immunization of Mouse

TGF-β1 was mixed with an equal volume of Complete Freund Adjuvant until the mixture became fluid and the resulting mixture was injected, in an amount of 100 μl/mouse, to the caudal vein of a 7 weeks-old Balb/c mouse. After 2 weeks, the same amount of TGF-β1 as in the first injection, which was mixed with Freund's incomplete adjuvant, was injected to the caudal vein of the mouse. After 4 to 5 days, a small amount of blood was taken from the tail and the presence of an antibody

for TGF-β1 was confirmed by ELISA. 30 μg of human TGF-β1 dissolved in 0.85% PBS was then intravenously injected 3 to 4 days before the following cell fusion procedure.

(Step 2) Cell Fusion

Myeloma cell SP2/0-Ag14(ATCC CRL 1581) was used as a mother cell in the cell fusion procedure. The mother cell was cultured in RPMI medium containing 10% FBS while maintaining a maximum cell density of $5x10^5$/ml.

The immunized mouse obtained in Step 1 was anesthetized using ether and its spleen was removed to be homogenized with a tissue homogenizer. The resulting homogenate was suspended in HBSS(Gibco-BRL, USA) and the resulting suspension was placed in a 15 ml centrifugal tube and centrifuged. This procedure was repeated twice to wash the spleen cells thoroughly. The mother cells, SP2/0-Ag14, were suspended in HBSS and centrifuged. This procedure was repeated twice. The spleen cells and the SP2/0-Ag14 cells were respectively resuspended in 10 ml of HBSS to count the cell number in each suspension. 10^8 spleen cells and 10^7 SP2/0-Ag14 cells taken from respective suspensions were mixed in a centrifugal tube and then centrifuged to precipitate the cells. The centrifugal tube was tapped with fingers to disperse the precipitated cells and, then, kept at 37 °C for 1 minute. 1 ml of HBSS containing 45% PEG(w/v) and 5% DMSO were added thereto over a period of 1 minute, followed by shaking the tube for 1 minute. 9 ml of RPMI medium was added thereto over a period of 3 minute and then RPMI medium was added thereto until the total volume of the cell suspension became 50 ml while shaking the tube. The resulting suspension was centrifuged and the cell pellet thus obtained was resuspended at a concentration of 1 to 2 x 10^5/ml in HAT medium(Gibco-BRL, USA). 0.2 ml

portions of the resulting resuspension were placed in the wells of a 96-well microtiter plate and then cultured for several days in an incubator, maintaining the condition of 37 °C, 5% CO_2 and 100 % humidity.

(Step 3) Selection of Hybridoma cell Producing Monoclonal antibody

The hybridoma cells obtained in Step 2 were subjected to ELISA using human TGF-β1 antigen to obtain cells which specifically react with TGF-β1, as described below.

Human TGF-β1 antigen was added to the wells of a microtiter plate in an amount of 50 μl(2 μg/ml)/well and kept at room temperature for 12 hours to attach the antigen to the well surface. The wells were washed with PBST to remove unattached antigen.

The hybridoma cell culture obtained in Step 2 was added in an amount of 50 μl/cell to each well and kept at 37 °C for 1 hour. The wells were washed with PBST to remove the culture. Goat anti-mouse IgG-HRP(sigma, USA) was added thereto, held at room temperature for 1 hour and washed with PBST. 100 μl of Substrate solution(OPD, Sigma) was added thereto, held at room temperature for 20 minutes, and the optical density of the resulting reaction mixture was measured at 492 nm.

Hybridoma cell lines secreting antibodies having high specificity for human TGF-β1 antigen were first selected, and each of these hybridoma cell lines was subjected to ELISA using human TGF-β1, -β2 and -β3 to screen hybridoma cells which have specificity only for human TGF-β1 antigen. Each of the hybridoma cells thus obtained was subjected to limiting dilution to obtain 7 hybridoma cell line clones producing a monoclonal antibody, hTGF-7, -8, -31, -46, -70, -119 and -207. Each clone was freeze-dried.

The hybridoma cell culture was centrifuged and the supernatant was subjected to ELISA to determine the antibody titer and then subjected to immunotype kit(Sigma, USA) to determine the subclass type of the antibody. The results are shown in Table V.

Table V

Clone No.	Optical Density(492 nm)	Subclass Type
hTGF-46	2.125	IgG1
HTGF-7	1.644	IgG1
HTGF-70	2.590	IgG1
HTGF-8	2.395	IgG1
HTGF-207	1.735	IgG1
HTGF-119	2.462	IgG1
HTGF-31	2.282	IgG1

As can be seen from Table V, all of the 7 clones were IgG1.

Among the 7 clones, the clone having the highest titer, hTGF-46, was selected and injected intraperitoneally to a mouse. Then its ascites was collected and subjected to western blotting. The results showed that the hybridoma cell line clone hTGF-46 secrets a monoclonal antibody having a high specificity for human TGF-β1. The hybridoma cell line hTGF-46 was deposited with Korean Collection for Type Culture(Address: #52, Oun-dong, Yusong-ku, Taejon 305-600, Republic of Korea) on April 20, 1998 under accession number of KCTC 0460BP, in accordance with the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganism for the Purpose of Patent Procedure.

Example 7: Production of Monoclonal Antibody for TGF-β1

To produce monoclonal antibody for TGF-β1 using the hybridoma cell line obtained in Example 6, 0.5 ml of

pristane was injected intraperitoneally to Balb/c mice, and after 1 week, $5x10^6$ hybridoma cells were injected to each mouse. From the mice having swollen abdominal cavity, ascites containing a high concentration of hybridoma cells was taken and centrifuged at 10,000 rpm to remove the hybridoma cells. The supernatant was stored at -20 °C.

Column was filled with protein G beads and then washed four times with 1xPBS. 2 ml of the supernatant was applied dropwise at a rate of 5 drops/minute to the column, and 0.1 M glycine-HCl solution was introduced at a rate of 1 drop/10 minutes to the column to elute IgG.

HRP was activated in 0.1 M sodium phosphate buffer(pH 6.5) containing 1.25 % glutaraldehyde and the activated HRP was dialyzed against carbonate buffer(pH 9.2). The dialyzed HRP was reacted with the IgG to obtain a HRP-conjugated IgG. After completion of the reaction, the RZ value(A_{403}/A_{280}) was determined by measuring the optical density of the reaction mixture at 280nm and 403 nm. In order to determine the activity of the enzyme-conjugated antibody, each well of a microtiter plate was coated with 1 μg of TGF-β1 and then reacted with the HRP-conjugated IgG to determine the activity. Futher, the HRP-conjugated IgG was subjected to Western blotting to confirm the activity thereof.

Example 8: Western Blotting

To examine whether the TGF-β1-specific monoclonal antibody obtained in Example 7 reacts with TGF-β2 and TFG-β3, the monoclonal antibody was subjected to SDS-PAGE and western blotting as follows.

Human TGF-β1, -β2 and -β3 proteins were subjected to SDS-PAGE on a 10% SDS-polyacrylamide gel and then transferred electrically to a nitrocellulose filter membrane. The membrane was reacted with the monoclonal

hours. The resulting membrane was treated with 3% bovine serum albumin at room temperature for 12 to 14 hours to block nonspecific reactions of the proteins. The membrane was washed three times with PBS containing 0.5% Tween 20 and then reacted with HRP-conjugated anti-mouse IgG(Sigma, USA) at room temperature. The membrane was washed with PBS containing 0.5% Tween 20, and then, a substrate solution(TMB, Gibco-BRL, USA) was added to the membrane to develop.

The result showed that the monoclonal antibody of the present invention reacts only with human TGF-β1 and did not reacted with human TGF-β2 and -β3. Therefore, the present monoclonal antibody has specificity only for human TGF-β1.

While the invention has been described with respect to the above specific embodiments, it should be recognized that various modifications and changes may be made to the invention by those skilled in the art which also fall within the scope of the invention as defined by the appended claims.

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

issued pursuant to Rule 7.1

TO: Choe, Yong-Kyung
ShindongA Apt. 1-203, # san 1 Yongjeon-dong, Dong-ku, Taejon 300-200, Republic of Korea

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: **hTGF - 46**	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: **KCTC 0460BP**
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by: [x] a scientific description [ ] a proposed taxonomic designation (Mark with a cross where applicable)	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on **April 30 1998.**	
IV. RECEIPT OF REQUEST FOR CONVERSION	
The microorganism identified under I above was received by this International Depositary Authority on and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: **Korea Research Institute of Bioscience and Biotechnology Korean Collection for Type Cultures** Address: KCTC, KRIBB #52 Oun-dong, Yusŏng-ku, Taejŏn 305-600, Republic of Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Kyung Sook Bae, Curator Date: **May 4 1998**

What is claimed is:

1. A method for quantifying the amount of TGF-β1 in a sample which comprises treating the sample with a TGF-β1-specific receptor to form a complex between TGF-β1 and the receptor and measuring the amount of the complex.

2. The method of claim 1, which comprises

(a) attaching the TGF-β1-specific receptor to a solid support;

(b) adding the sample to the supported receptor to form the TGF-β1-receptor complex;

(c) binding a TGF-β1-specific antibody conjugated with a label to the complex; and

(d) measuring the amount of TGF-β1 using the label as a detection marker.

3. The method of claim 1 or 2, wherein the TGF-β1-specific receptor is TGF-β1 type III receptor.

4. The method of claim 2, wherein the TGF-β1-specific antibody is an antibody prepared by immunizing a mammal with TGF-β1 or a part thereof.

5. The method of claim 4, wherein the antibody is a monoclonal antibody or polyclonal antibody.

6. The method of claim 5, wherein the monoclonal antibody is produced from a hybridoma cell line hTGF-46(KCTC 0460BP).

7. The method of claim 1 or 2, wherein the concentration of TGF-β1 in the sample is 30 pg/ml or lower.

8. The method of claim 2, wherein the label is horseradish peroxidase, biotin or fluorescence.

9. A method for detecting a cancer in a patient which comprises treating a body fluid sample taken from the patient with a TGF-β1-specific receptor to form a complex between the receptor and TGF-β1 present in the sample; measuring the amount of the complex to quantify the concentration of TGF-β1 in the sample; and comparing the TGF-β1 concentration with that of a healthy person.

10. The method of claim 9, which comprises

(a) attaching the TGF-β1-specific receptor to a solid support;

(b) adding the sample to the supported receptor to form the TGF-β1-receptor complex;

(c) binding a TGF-β1-specific antibody conjugated with a label to the complex;

(d) measuring the amount of TGF-β1 using the label as a detection marker to determine the TGF-β1 concentration in the sample; and

(e) comparing the TGF-β1 concentration with that of a healthy person.

11. The method of claim 9 or 10, wherein the TGF-β1 specific receptor is TGF-β1 type III receptor.

12. The method of claim 10, wherein the TGF-β1-specific antibody is an antibody prepared by immunizing a mammal with TGF-β1 or a part thereof.

13. The method of claim 12, wherein the antibody is a monoclonal antibody or a polyclonal antibody.

14. The method of claim 13, wherein the monoclonal antibody is produced from a hybridoma cell

line hTGF-46(KCTC 0460BP).

15. The method of claim 9 or 10, wherein the concentration of TGF-β1 in the sample is 30 pg/ml or lower.

16. The method of claim 10, wherein the lable is horseradish peroxidase, biotin or fluorescence.

17. The method of claim 9 or 10, wherein the cancer is selected from the group consisting of stomach cancer, hepatoma, breast cancer, lung cancer, rectal-colic cancer, prostate cancer and uterine cervical cancer.

18. The method of claim 9 or 10, wherein the body fluid is plasma or urine.

19. A composition for detecting a cancer which comprises a TGF-β1-specific receptor and a TGF-β1-specific antibody.

20. The composition of claim 19, wherein the TGF-β1 specific receptor is TGF-β1 type III receptor.

21. A hybridoma cell line which is hTGF-46(KCTC 0460BP) producing a human TGF-β1-specific monoclonal antibody.

22. A TGF-β1-specific monoclonal antibody produced by hybridoma cell line hTGF-46(KCTC 0460BP).

Fig. 1

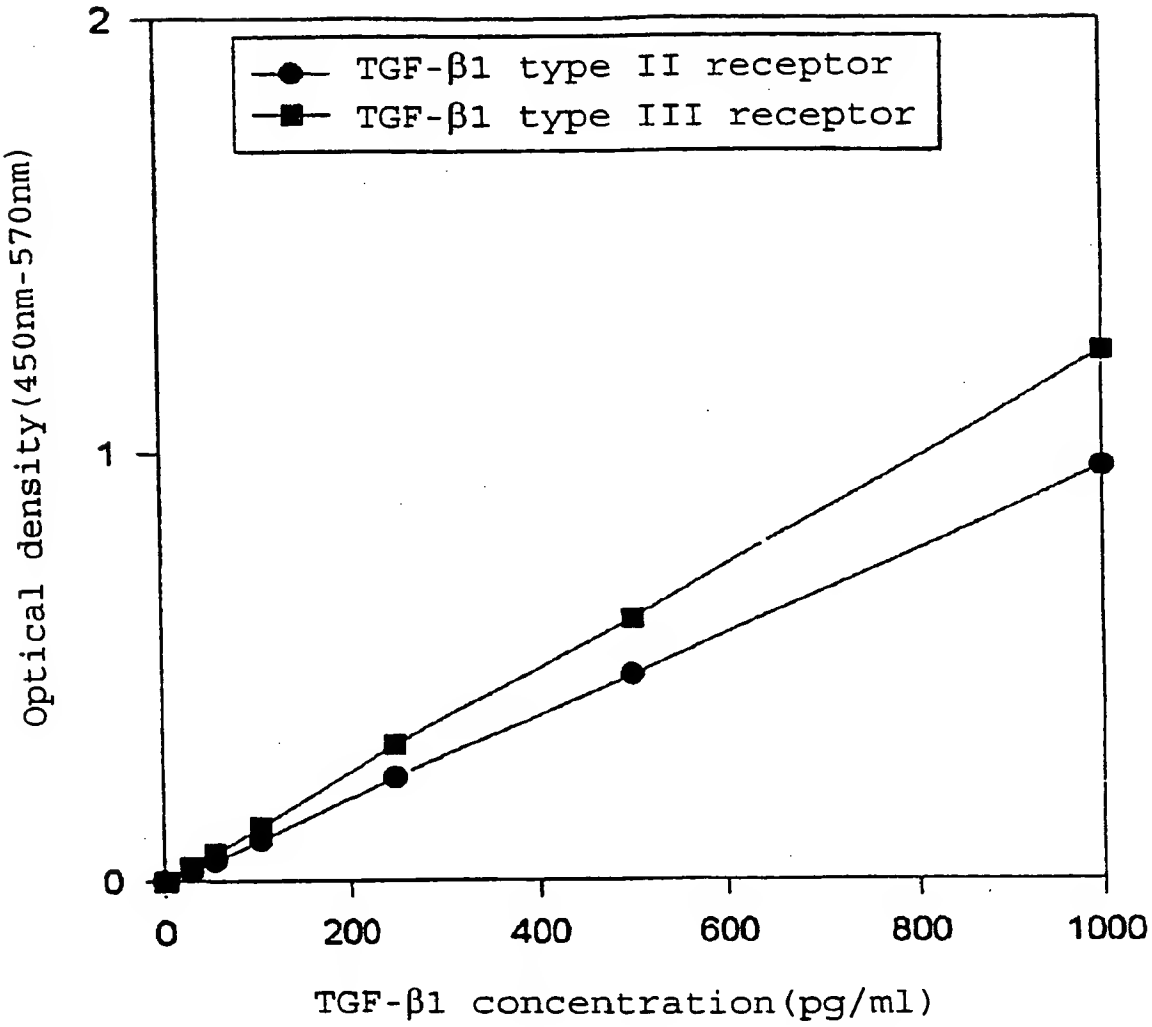


Fig. 2

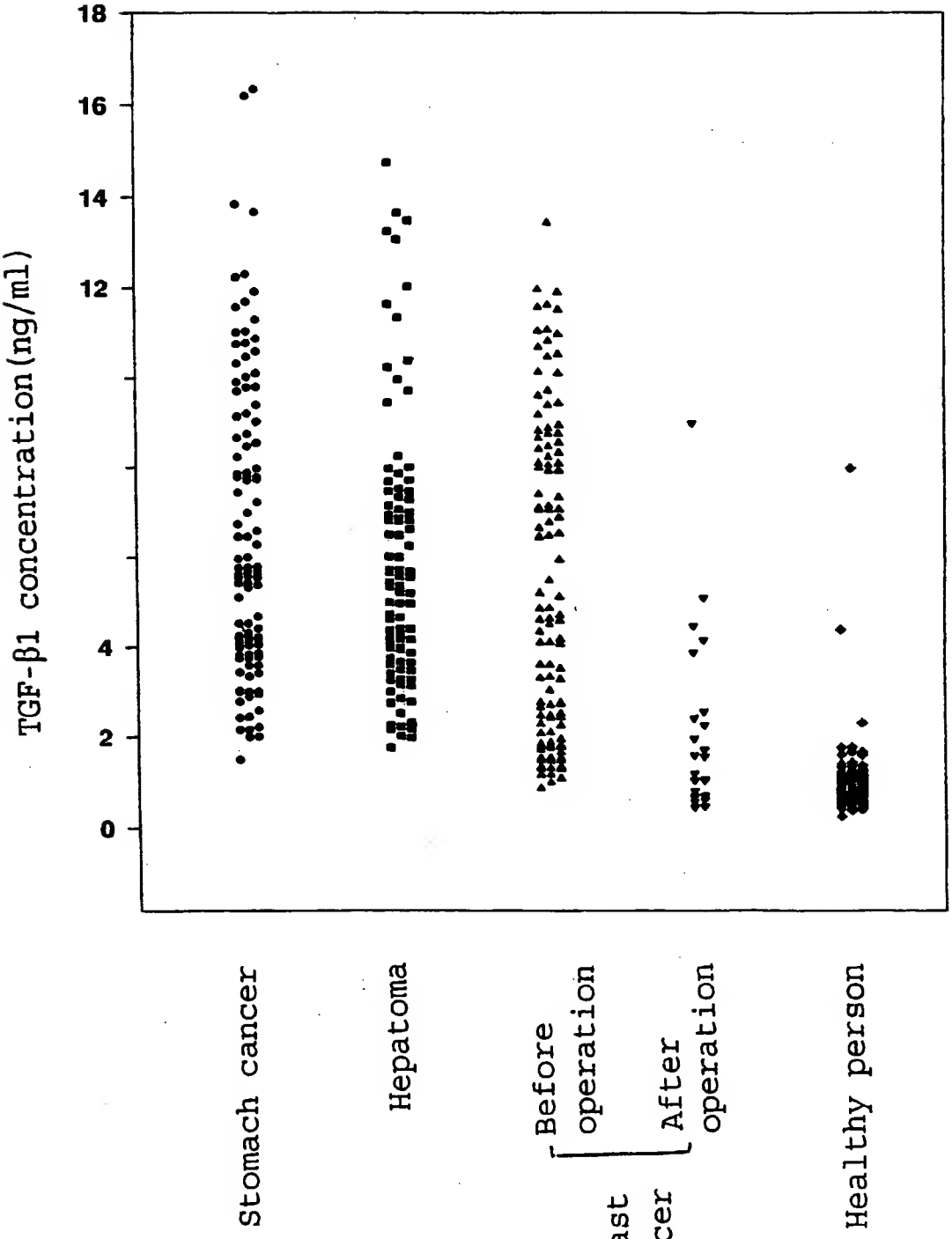
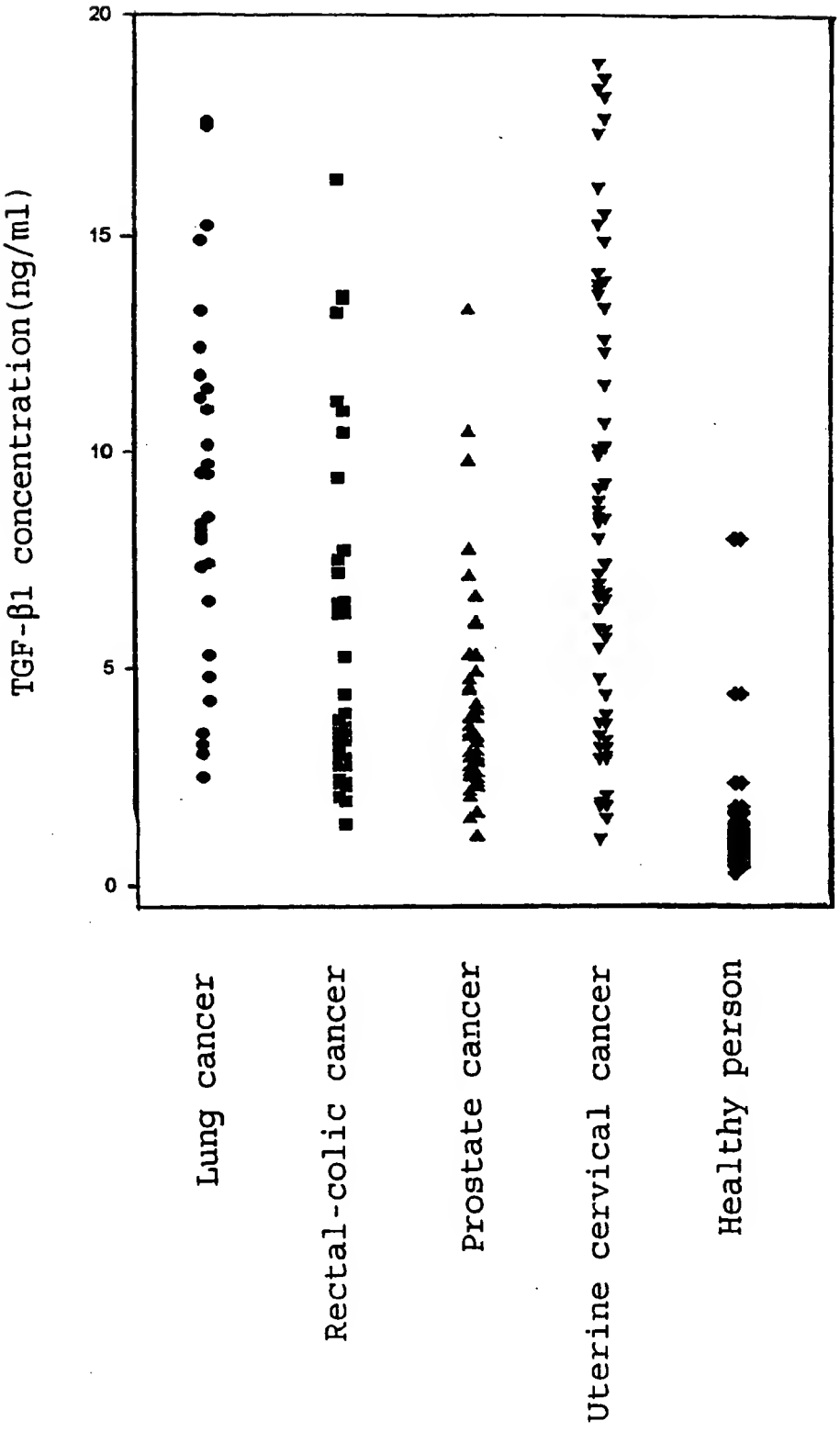


Fig. 3



Sequence Listing

<110> Hanmi Pharm. Co., Ltd

<120> METHOD FOR QUANTIFYING TRANSFORMING GROWTH FACTOR-β1 AND METHOD FOR DETECTING CANCER BY USING SAME

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR00/00329

A. CLASSIFICATION OF SUBJECT MATTER

IPC7 G01N 33/53

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimun documentation searched (classification system followed by classification symbols)

IPC7 G01N 33/53, G01N 33/577, C12P 21/08, C12Q 1/00

Documentation searched other than minimun documentation to the extent that such documents are included in the fileds searched

Korean Patents and applications for inventions since 1975
Korean Utility models and applications for Utility models since 1975
Japanese Utility models and applications for Utility models since 1975

Electronic data base consulted during the intertnational search (name of data base and, where practicable, search trerms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JP 02-126157(TOSOH CORP) 15 MAY 1990 - Claim and abstract	1
A	JP 59-226864(TOYO JOZO CORP; NIPON CHEMICAL RESEARCH CORP) 20 DECEMBER 1984 - See the all document	1-22
A	JP 59-195162(TOYO JOZO CORP; NIPON CHEMICAL RESEARCH CORP) 06 NOVEMBER 1984 - See the all document	1-22
A	JP 59-216058(TOYO JOZO CORP; NIPON CHEMICAL RESEARCH CORP) 06 DECEMBER 1984 - See the all document	1-22
A	JP 60-018764(TOYO JOZO CORP; NIPON CHEMICAL RESEARCH CORP) 3 0 JANUARY 1985 - See the all document	1-22

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevence
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevence; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevence; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
08 AUGUST 2000 (08.08.2000)	14 AUGUST 2000 (14.08.2000)

Name and mailing address of the ISA/KR
Korean Industrial Property Office
Government Complex-Taejon, Dunsan-dong, So-ku, Taejon
Metropolitan City 302-701, Republic of Korea
Facsimile No. 82-42-472-7140

Authorized officer
JOO, Young Sik
Telephone No. 82-42-481-5995

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR00/00329

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 1-18
because they relate to subject matter not required to be searched by this Authority, namely:
Although claim 1 to 18 is directed a method of diagnostic, the search has been carried out and based on the alleged effects of composition.

2. [ ] Claims Nos.:
because they relate to part of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Search Authority found multiple inventions in this international application, as follows:

1. [ ] As all required addtional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be established without effort justifying an additional fee, this Authority did not invite payment of any addition fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/KR00/00329

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
JP 02-126157	15, 05, 1990	NONE	
JP 59-226864	20, 12, 1984	NONE	
JP 59-195162	06, 11, 1984	NONE	
JP 59-216058	06, 12, 1984	NONE	
JP 60-018764	30, 01, 1985	NONE	